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Lyn Tyrosine Kinase: Accentuating the Positive and the Negative

Review

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Summary

Lyn, one of several Src-family tyrosine kinases in immune cells, is noted for its ability to negatively regulate signaling pathways through phosphorylation of inhibitory receptors, enzymes, and adaptors. Somewhat paradoxically, it is also a key mediator in several pathways of B cell activation, such as CD19 and CD180. Whether Lyn functions to promote or inhibit immune cell activation depends on the stimulus and the developmental state, meaning that the consequences of Lyn activity are context dependent. The importance of regulating Lyn activity is exemplified by the pathological conditions that develop in both *lyn*$^{-/-}$ and *lyn* gain-of-function mice (*lyn*$^{up/up}$), including lethal antibody-mediated autoimmune diseases and myeloid neoplasia. Here, we review the outcomes of altered Lyn activity within the framework of B cell development and differentiation and the circumstances that appear to dictate the outcome.

Introduction

The challenge for molecular immunology is to determine how lymphocyte surface receptors that interact with antigens transmit their signals into the nucleus to elicit a stimulus that is appropriate to maintain the well being of the host. Examination of normal and abnormal responses has helped develop an understanding of the molecular and cellular processes that govern the differentiation of lymphocytes. The Src-family kinase (SFK) Lyn has unique regulatory properties such that alterations in its activity or expression profoundly alter the functioning of the immune system. Mice lacking Lyn or expressing a constitutively active form of Lyn transform the humoral immune system from being self-protective to self-destructive. Clearly, processes modulated by or dependent on Lyn are critical to the immune system's ability to appropriately discern self from nonself.

Lyn is the predominant SFK in B cells, and based on numerous biochemical tests, Lyn was initially assigned a role in signal initiation. This was in keeping with both the data and the general expectation of the function of SFK in signal transduction. It was not until *lyn*$^{-/-}$ mice were found to succumb to autoimmune disease and *lyn*$^{-/-}$ B cells were found to be hyperresponsive to B cell receptor (BCR) ligation that the role of Lyn as a negative regulator of signal transduction was revealed. These seminal findings in B cells led to an understanding of the function of Lyn in multiple cell types and pathways. Indeed, the diversity of Lyn's targets has made it difficult to determine the key changes associated with the pathology in knockout mice. Stimulatory roles for Lyn in B cells have also been defined and although few appear to be unique, dysregulation can also result in disease. It is possible that through an assessment of Lyn-dependent biochemical and cellular processes in B cell development, pathways that contribute to immune dysregulation and disease susceptibility will be identified.

A Brief History of Lyn in B Cells

The Src family of nonreceptor protein tyrosine kinases comprises of Lyn, Src, Yes, Fgr, Fyn, Lck, Hck, Blk, and Yrk (Brown and Cooper, 1996). The *lyn* gene is localized on human chromosome 8q13 (Yamanashi et al., 1987) and mouse chromosome 4qA1, although in mouse, a pseudogene is present (Hibbs et al., 1995a). Lyn exists in two isoforms, p53 and p56, arising from alternate splicing of exon 2 (Stanley et al., 1991; Yi et al., 1991), but no functional differences between isoforms are currently known. *lyn* is expressed in all blood cells except T cells, although *lyn*-expressing HTLV-transformed human T cells have been reported (Yamanashi et al., 1989). SFK structure and regulation have been reviewed recently (Lowell, 2004) and are summarized in Figure 1.

Lyn is associated with BCR complexes containing IgM or IgD (Yamanashi et al., 1991) as well as the B cell costimulatory molecule CD19 (van Noesel et al., 1993). Lyn is rapidly phosphorylated and activated upon BCR crosslinking (Burkhardt et al., 1991), which correlates with the tyrosine phosphorylation of Igα/β (Saouaf et al., 1994), suggesting a causative relationship. A *lyn*$^{-/-}$ variant of the chicken B cell line DT40 exhibited delayed calcium influx after BCR ligation (Takata et al., 1994), indicating a requirement for Lyn in promoting BCR-induced calcium mobilization, although this data must be viewed in the context of Lyn being the sole SFK in these cells (Takata et al., 1994). Lyn was found to interact with and phosphorylate tyrosine residues in Btk and Syk kinases, HS1 protein, and the Cbl proto-oncogene product (reviewed in Gauld and Cambier [2004] and Lowell [2004]). After BCR engagement, Lyn and Fyn also associate with the p85 subunit of phosphoinositide-3 kinase (PI3K), an association that increases PI3K activity (Pleiman et al., 1994). Lyn can also phosphorylate tyrosine(s) in the cytoplasmic domain of CD19 and in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the Igα/Igβ subunits (Gauld and Cambier, 2004; Lowell, 2004). These data implied a prominent role for Lyn in the initiation of signals from the BCR and its costimulators, making it quite surprising when *lyn*$^{-/-}$ mice developed a lethal antibody-mediated glomerulonephritis (Hibbs et al., 1995b; Nishizumi et al., 1995). This phenomenon remained to a large extent inexplicable until *lyn*$^{-/-}$

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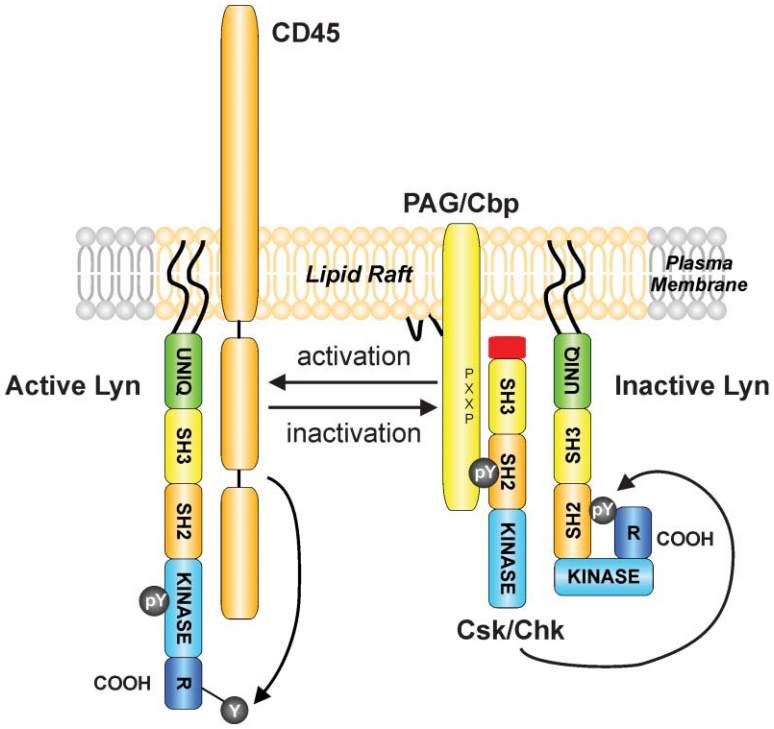

Figure 1. Lyn Structure and Mode of Regulation

Lyn is both myristylated and palmitylated localizing it to lipid rafts within the plasma membrane (indicated by the lighter shading of the plasma membrane). All SFKs are held in an inactive state through phosphorylation of a tyrosine residue in their C-terminal regulatory (R) domain by Csk family enyzmes. Csk is itself regulated by membrane localization through interaction with PAG/Cbp. Phosphorylation of the C-terminal tyrosine in SFKs holds the enzymes in a closed conformation through SH2 domain interactions. Lyn can be activated by dephosphorylation of the C-terminal regulatory tyrosine by CD45. CD45 is shown within the lipid raft and acting to dephosphorylate Y508 for graphic purposes. Its location varies over time and it is also able to dephosphorylate Y397, inhibiting SFK activity (Ashwell and D'Oro, 1999).

B cells were found to be both hyperresponsive to BCR ligation and resistant to the inhibitory consequences of coligating FcγRIIb1 or CD22 with the BCR (Chan et al., 1997, 1998a; Cornall et al., 1998; Nishizumi et al., 1998; Smith et al., 1998). Thus, Lyn was revealed as a critical negative regulator of B cell activation.

B Cell Development

Given the clear role for Lyn in signal transduction from the BCR of mature B cells, it is reasonable to ask what role it plays in the early stages of B cell development and how such roles may differ from those in mature B cells (Figure 2). It is somewhat surprising to find that Lyn appears to play only a minor, or at least redundant, role in pro- and pre-B cells. Although the absence of Lyn alters the cycling status of multipotent hematopoietic precursors (Harder et al., 2004), there is no indication that this persists into the early stages of the B cell lineage. In the absence of Lyn or presence of constitutively active Lyn, pro-B cells are both normal in phenotype and frequency when measured as a proportion of total bone marrow (BM) (Chan et al., 1997; Hibbs et al., 1995b, 2002; Nishizumi et al., 1995), suggesting that Lyn does not control the proliferation or survival of pro-B cells. A clear role for Lyn in the pre-B stage is only apparent in combination with Fyn and Blk deficiency; progression of B lineage cells lacking these three SFK is greatly diminished beyond the pre-B stage (Saijo et al., 2003). This block is independent of Syk and not related to phosphorylation of pre-BCR proximal signaling molecules such as phospholipase C-γ2 (PLC-γ2) and Vav (Saijo et al., 2003). The current explanation is that signaling through the pre-BCR in the absence of the three SFKs fails to activate the atypical PKCλ and hence IKKα, IKKβ, and ultimately NF-κB (Saijo et al., 2003); however, NF-κB-deficient mice do not show a similar phenotype (Scott et al., 1993). Indeed, the normal pre-BCR proximal

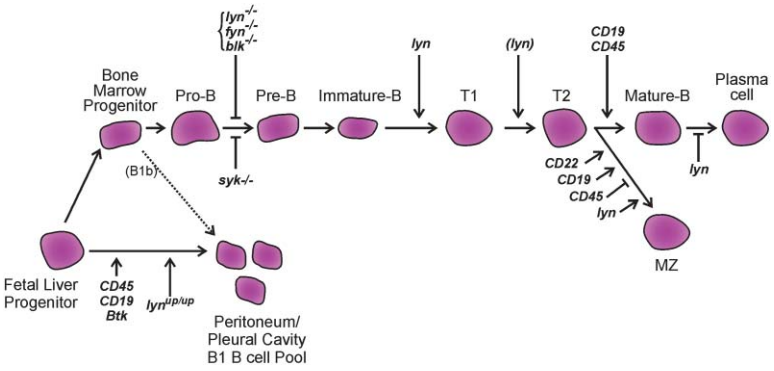

Figure 2. Scheme of B Cell Development and the Importance of Lyn and Lyn-Regulated Proteins in this Process

Conventional B cells arise from fetal liver and adult BM, whereas B1a B cells originate in fetal omentum and are thought to play an important role in innate immunity, secreting large amounts of low-affinity IgM antibody. CD45 and CD19 are required for B1 B cell development, and constitutively active Lyn expands this compartment. SFK are critical for conventional B cell development beyond the pro-B cell stage as *lyn*$^{-/-}$*fyn*$^{-/-}$*blk*$^{-/-}$ mice are blocked at this point. Lyn and Lyn targets have important and unique roles beyond the immature B cell developmental stage, arrows show where they enhance and bars where they inhibit a developmental step. Lyn is also critical for tempering plasma cell differentiation as *lyn*$^{-/-}$ mice show increased frequency of plasma cells. Marginal zone (MZ) B cell generation is blocked in both *lyn*$^{-/-}$ and *lyn*$^{up/up}$ mice.

signal transduction in the absence of these SFKs suggests the defect may be in cell survival. Irrespective of the molecular basis of the developmental arrest, it remains that Lyn contributes a positive role early in B cell development.

Completion of IgH gene rearrangement coincides with the first manifestation of Lyn as a negative regulator of B cell activity. Although the developmentally earliest of these examples is relatively indirect, it serves to resolve the role of Lyn from that of other SFKs. More frequent Igκ gene rearrangement has been observed in the absence of Lyn and presence of an Igλ transgene (Meade et al., 2002), suggesting that Lyn may contribute to allelic exclusion at the IgL loci. Although this has not been noted in other Ig transgenic systems (Cornall et al., 1998; Seo et al., 2001), Lyn may well have a role in mediating allelic exclusion as other components of the BCR have been reported to be involved (Meade et al., 2004; Schweighoffer et al., 2003).

Although *lyn*$^{-/-}$ mice contain essentially normal B lineage compartments through to the transitional stage in spleen (Chan et al., 1997; Hibbs et al., 1995b; Nishizumi et al., 1995), introducing Ig transgenes and their neoself determinants onto the *lyn*$^{-/-}$ background revealed Lyn as a negative regulator of BCR signaling in immature B cells in the BM (Cornall et al., 1998). For example, hen egg lysozyme (HEL)-reactive *lyn*$^{-/-}$ B cells are deleted at this stage in the BM in the presence of soluble HEL, whereas control B cells survive and enter the periphery as anergic (Cornall et al., 1998). Thus, as for B cells lacking the phosphatase SHP-1 (Cyster and Goodnow, 1995), Lyn deficiency increases sensitivity to tolerance induction in the BM. Curiously, an independent transgenic self-reactive system specific for DNA did not show a similar effect in the BM (Seo et al., 2001), highlighting the potential contribution of either the antigen, the genetic background, or both. One may speculate from the numerically normal immature B cell compartment in the BM of nontransgenic *lyn*$^{-/-}$ mice (Allman et al., 2001; Chan et al., 1997; Hibbs et al., 1995b; Nishizumi et al., 1995) that any hyperresponsiveness of the BCR is compensated by a shift in the repertoire.

Analysis of *lyn*$^{-/-}$ B cell subsets in the periphery has identified this as a critical point requiring normal Lyn activity. Although the number of immature B cells in *lyn*$^{-/-}$ BM is normal (Chan et al., 1997; Hibbs et al., 1995b; Nishizumi et al., 1995), there is a significant deficit in transitional B cells in the spleen (Allman et al., 2001; Meade et al., 2002). When that compartment is subdivided, the defect appears to be in entry into the T1, 2, and 3 stages (Allman et al., 2001). However, this conclusion is not unanimous because a developmental block between T1 and T2 that is surmountable by a *bcl-2* transgene has been reported (Meade et al., 2002), suggesting that Lyn transmits a positive or survival signal for B cell recruitment into the periphery, similar to that of Syk (Turner et al., 1997). Although this result is consistent with a requirement for phosphorylation of Igα—a known target of Lyn—for B cell survival (Torres et al., 1996), it is difficult to reconcile with the negative role identified for Lyn in immature BM B cells (Cornall et al., 1998). Possible explanations are that Lyn has diametrically opposite roles in successive stages of B cell development, that these observations are actually both manifestations of enhanced deletion of self-reactive B cells, or that the substrates targeted by Lyn vary with the degree or nature of BCR stimulation.

There is agreement that *lyn*$^{-/-}$ mice suffer from a deficit of mature B cells in the periphery and this is apparent in the marginal zone (MZ) and follicular compartments of spleen and in recirculating B cells in lymph nodes and BM (Chan et al., 1997; Hibbs et al., 1995b; Nishizumi et al., 1995). Alteration of the number of MZ B cells is considered to reflect changed BCR signaling thresholds, a phenomenon also seen in *CD19*$^{-/-}$ and *Aiolos*$^{-/-}$ mice (Engel et al., 1995; Rickert et al., 1995; Wang et al., 1998). The loss of mature *lyn*$^{-/-}$ B cells has been attributed to enhanced apoptosis and to the rapid differentiation of mature B cells into antibody-secreting cells (ASC) (Chan et al., 1997). Although a *bcl-2* transgene increased the number of B cells in the periphery of *lyn*$^{-/-}$ mice, it neither altered the maturation state nor raised the B cell number to that seen in control *bcl-2* transgenic mice (Meade et al., 2002; M. Janas, M.L.H., and D.M.T., unpublished data), indicating that impaired cell survival is not the only defect.

The B1 B cell compartment remains controversial. Early reports indicated that the majority of cells in the enlarged spleens of *lyn*$^{-/-}$ mice were Mac-1^{+} cytoplasmic IgM^{+} (Chan et al., 1997; Nishizumi et al., 1995), classifying these cells as belonging to the B1 lineage, although splenic B1 cells typically do not express Mac-1 (Hardy and Hayakawa, 2001). Other studies, however, have reported either unchanged or a modest diminution in B1a B cell numbers in *lyn*$^{-/-}$ mice (Hasegawa et al., 2001; Hibbs et al., 1995b; Satterthwaite et al., 1998; Whyburn et al., 2003). This analysis is complicated by the dramatically increased numbers of erythroid and Mac-1^{+} myelomonocytic cells and their progenitors in *lyn*$^{-/-}$ spleen (Harder et al., 2001, 2004; Satterthwaite et al., 1998). Superficially, a linkage between enhanced B1 B cells and autoimmune disease in *lyn*$^{-/-}$ mice would fit with increases in this subpopulation in autoimmune prone strains such as *NZB* and *motheaten*. However, *lyn*$^{-/-}$ BM efficiently transfers anti-nuclear antibody development into irradiated recipients (K.W.H., D.M.T., and M.L.H., unpublished data), indicating that B1a cells are not the source of these antibodies as BM transfers these cells poorly (Hardy and Hayakawa, 2001).

The final developmental compartment to be considered is that of ASC. Analysis of serum Ig titers in *lyn*$^{-/-}$ mice revealed a 10-fold increase in IgM (Hibbs et al., 1995b; Nishizumi et al., 1995), with an equivalent increase in IgM ASC in all lymphoid tissues (Hibbs et al., 1995b), although in adult *lyn*$^{-/-}$ mice, the frequency of IgG ASC and IgG levels were normal (Hibbs et al., 1995b; Janas et al., 1999; Nishizumi et al., 1995). Although there is general agreement with these observations, there is little understanding of the underlying cause. Initial reports suggested that the expansion in ASC was due to an expanded B1 population (Chan et al., 1997; Nishizumi et al., 1995), but this observation remains contentious (see above). An alternative is that the intrinsic BCR hyperresponsiveness of *lyn*$^{-/-}$ B cells predisposes them to differentiate (Chan et al., 1997). It could be argued that the tonic signaling required to sustain mature B cells (Lam et al., 1997) is amplified or misinterpreted by *lyn*$^{-/-}$ B cells such that they differentiate into ASC at

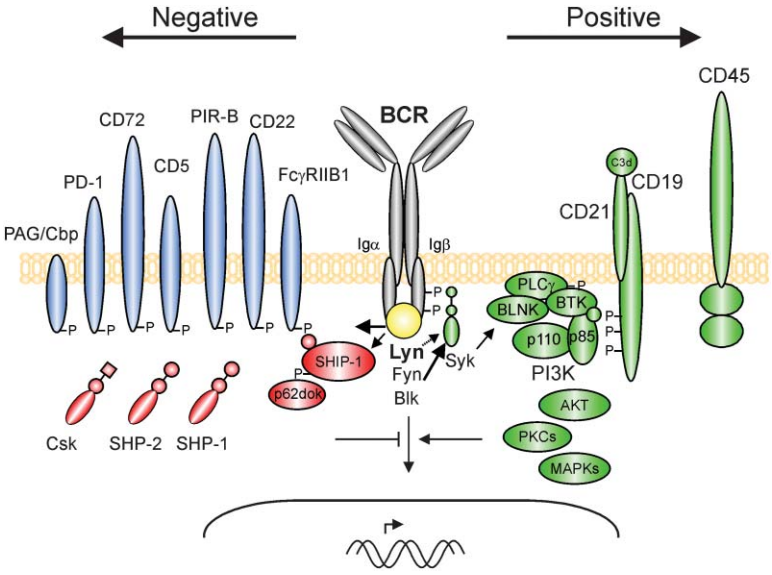

Figure 3. Schematic Representation of Signal Transduction Pathways Modulated by Lyn in B Cells

Lyn contributes to positive regulation of signaling through tyrosine phosphorylation of ITAMs in the Igα and Igβ components of the BCR and in CD19. This role can be assumed by other SFKs such as Fyn and Blk, and promotes B cell activation through the recruitment of Syk and PI3K. Lyn plays an essential role in negative regulation of signaling through its unique ability to phosphorylate ITIMs in inhibitory cell surface receptors such as FcγRIIb1, CD22, PIR-B, and CD5 and a potential inhibitory site on Syk (see text). Whether CD72, PAG, and PD-1 are Lyn targets in vivo has yet to be confirmed. Positive phosphorylation of Syk is redundant with other SFKs. ITIM phosphorylation induces the recruitment of inhibitory phosphatases such as SHP-1/2 and SHIP-1, which down modulate signaling. Lyn is also suspected to be critical for the phosphorylation of PAG/Cpb in B cells, thereby enabling Csk recruitment to the plasma membrane where it may modulate SFK activity. Squares, SH3 domains; circles, SH2 domains; and −P, phospho tyrosines. The single −P on each inhibitory receptor is indicative and not a representation of the true target number.

a high frequency. The fact that restricting the B cell population to a single specificity by transgenesis does not normalize the rate of differentiation (Cornall et al., 1998; Meade et al., 2002) indicates that specificity alone is not the key determinant. Significantly, the DNA-reactive Ig transgenic *lyn*$^{-/-}$ model showed greatly diminished ASC formation (Seo et al., 2001) as did *lyn*$^{-/-}$ mice carrying a hypomorphic allele of *Btk* (Whyburn et al., 2003), suggesting that over production of ASC is responsive to BCR signaling, possibly transmitted through Btk.

Amplification of BCR Signaling by Lyn

B cells from *lyn*$^{-/-}$ mice showed essentially normal initiation of BCR signaling, albeit delayed (Chan et al., 1997), indicating no absolute requirement for Lyn in these processes. The involvement of Lyn in BCR signal amplification, however, is made clear from two studies, whereas unique roles for Lyn in positive signaling have been found outside the strict confines of the BCR, all with implications for autoimmunity (Figure 3).

lyn$^{up/up}$ *Mice*. B cells from *lyn*$^{up/up}$ mice, homozygous for a targeted Y508F mutation that results in sustained activation of the kinase, show constitutive tyrosine phosphorylation of positive signaling molecules, including Syk and PLC-γ2, and display a heightened calcium flux after BCR stimulation (Hibbs et al., 2002). Possibly to compensate for elevated baseline signaling, *lyn*$^{up/up}$ B cells down regulate surface IgM, CD45R, and the complement receptor CD21 (Hibbs et al., 2002). Although conventional B cell numbers are reduced, B1 B cells are increased, perhaps best explained by excess positive, rather than negative, signals. Furthermore *lyn*$^{up/up}$ mice develop circulating autoantibodies and a lethal autoimmune glomerulonephritis pathologically distinct from that of *lyn*$^{-/-}$ mice (Hibbs et al., 2002), again consistent with the net change in B cell signaling being toward activation. The situation, however, is quite complex as there is also constitutive activation of inhibitory pathways in *lyn*$^{up/up}$ B cells (Hibbs et al., 2002). *lyn*$^{up/up}$ peripheral B cells fail to proliferate after BCR stimulation, suggesting they are in an unstable equilibrium, but why this breaks down in favor of activation over inhibition is a critical question. Curiously, mice expressing a similarly mutated version of Blk, introduced as a B and T lineage restricted transgene, have no reported predisposition to autoimmunity but rather develop B lymphoid tumors (Malek et al., 1998): no B cell tumors have arisen in *lyn*$^{up/up}$ mice (K.W.H., D.M.T., and M.L.H., unpublished data). The different outcomes for Lyn- and Blk-gain-of-function mutations may reflect differences in level, onset, or tissue distribution or different roles for these SFK in B cell signaling.

The CD19-Lyn Amplification Loop. A second positive role reported for Lyn is through its interaction with CD19, which together with CD21, CD81, and Leu 13 forms a coreceptor complex that reduces the threshold of BCR activation by up to 10^4-fold (Fearon and Carroll, 2000). Although CD19 is considered critical to B cell activation due to association with Vav and PI3K (Wang et al., 2002), it also associates with Lyn (van Noesel et al., 1993) and Fyn (Chalupny et al., 1995). Recently, CD19 and Lyn were reported to form a signal amplification loop in mouse B cells in a process implying interdependence of Lyn and CD19 activity (Fujimoto et al., 2000). The phenotypes of lyn$^{-/-}$ and CD19$^{-/-}$ primary B cells do, however, differ in both B cell biology and biochemistry (Chan et al., 1997; Engel et al., 1995; Hibbs et al., 1995b; Nishizumi et al., 1995; Rickert et al., 1995), and there is also biochemical evidence showing Lyn and CD19 to be independent (Xu et al., 2002), leaving this issue unresolved. CD19 phosphorylation requires SFK activity but which doesn't seem to matter (Xu et al., 2002), and CD19 is constitutively phosphorylated in B cells from lyn$^{up/up}$ mice (Hibbs et al., 2002), indicating that Lyn can phosphorylate CD19. One must conclude that these differences in the Lyn dependence of CD19 phosphorylation reflect the efficiency of activating the SFK remaining in lyn$^{-/-}$ B cells by the stimulus in use. This in turn might reflect the stimulus itself or possibly the nature of the B cells,

which may alter as lyn$^{-/-}$ mice age (Nishizumi et al., 1998). It is interesting to speculate, however, that the constitutive phosphorylation of CD19 in lyn$^{up/up}$ B cells (Xu et al., 2002) may contribute to the autoimmune disease that develops in these mice (Hibbs et al., 2002), given the documented association between elevated CD19 and autoimmune disease (Sato et al., 2000; Tedder et al., 1997).

RP105 and CD38. Two further examples of positive signaling by Lyn in B cells are in the response to ligation of RP105 (CD180) and CD38 (Chan et al., 1998b; Yasue et al., 1997). *lyn*$^{-/-}$ B cells fail to proliferate after RP105 ligation, which is mitogenic for control B cells. RP105 activates Lyn, PKCbI/II, and MEK (Chan et al., 1998b) and forms part of the LPS receptor on B cells, acting with TLR4 in a MyD88-independent manner (Ogata et al., 2000; Yazawa et al., 2003). This complex is reported to recruit CD19, which is associated with Lyn and Vav (Yazawa et al., 2003). Although RP105's dependence on Lyn could be via CD19, this is unlikely as RP105 stimulation induces no proliferation of *lyn*$^{-/-}$ B cells (Chan et al., 1998b), whereas CD19$^{-/-}$ cells proliferate at 30% of control levels (Yazawa et al., 2003), suggesting a direct relationship between Lyn and RP105. Coligation of CD38 in the presence of IL5, although long recognized as being mitogenic, is of uncertain physiological relevance (Cockayne et al., 1998). It is, however, a Lyn-dependent process with proliferation of B cells lacking Lyn, Fyn, or both being greatly reduced. Lyn's unique role is the activation of Btk, whereas Lyn and Fyn cooperate in inducing optimal expression of the IL5Rα chain (Yasue et al., 1997).

Negative Regulation of BCR Signaling By Lyn

Generation of *lyn*$^{-/-}$ mice, and the discovery of their inherent predisposition to autoimmune disease and B cell hyperresponsiveness revealed a role for Lyn in the negative regulation of BCR signaling. Experiments in vitro showed that besides hyperproliferation in response to BCR crosslinking, *lyn*$^{-/-}$ primary B cells also showed elevated MAPK activation, enhanced calcium flux, and hyperactivation of Akt. These results define multiple mechanisms for Lyn in establishing negative feedback control of BCR signaling (Figure 3). As the biochemistry of the predominant inhibitory activities of Lyn in B cells has been recently reviewed (Gauld and Cambier, 2004; Lowell, 2004), this review focuses more on the cell biology and the contribution these pathways may make to the *lyn*$^{-/-}$ phenotype.

Phosphorylating Inhibitory Receptors

FcγRIIb1. Coligation of the BCR with the receptor for the Fc portion of immunoglobulin, FcγRIIb1, has long been known to inhibit B cell activation (Phillips and Parker, 1984). Indeed, FcγRIIb1-deficient mice exhibit elevated antibody responses (Takai et al., 1996) and develop rheumatoid arthritis and fatal glomerulonephritis on the C57BL/6 background (Bolland and Ravetch, 2000; Yuasa et al., 1999), just as many autoimmune-prone strains are genetically unable to upregulate FcγRIIb1 expression after stimulation (Pritchard et al., 2000). Lyn is required for tyrosine phosphorylation of FcγRIIb1 and lyn$^{-/-}$ B cells are resistant to the inhibitory effects of crosslinking FcγRIIb1 with the BCR (Chan et al., 1998a; Nishizumi et al., 1998). SHIP-1 recruitment to FcγRIIb1 is phosphorylation dependent (Ono et al., 1996) and therefore presumably defective in lyn$^{-/-}$ B cells. Lyn is also required for SHIP-1 phosphorylation (Hibbs et al., 2002), an event required to recruit p62dok and its associated RasGAP, which act to antagonize the Ras pathway (Tamir et al., 2000; Tridandapani et al., 1998). The fact that many of the regulatory functions of FcγRIIb1 require Lyn activity makes this connection a potentially strong predisposing factor to the development of autoimmune disease.

CD22. CD22, a B cell-restricted member of the sialoadhesin family of molecules, is rapidly phosphorylated on tyrosine after BCR ligation, facilitating recruitment of numerous intracellular signaling molecules including SHP-1, Lyn, Syk, PI3K, and PLC-γ (reviewed in Tamir and Cambier [1998]). CD22 is primarily an inhibitory receptor, as B cells from CD22$^{-/-}$ mice show enhanced BCR-induced Ca^{2+} flux (O'Keefe et al., 1996; Otipoby et al., 1996), whereas the activation of MAPK pathways is suppressed when CD22 is coligated with the BCR but enhanced when CD22 is separately ligated before BCR engagement (Tooze et al., 1997). On the appropriate genetic background and with age, CD22$^{-/-}$ mice develop high titers of IgG anti-DNA antibodies but without any apparent pathology (O'Keefe et al., 1999). Four studies showed that CD22, Lyn, and SHP-1 form a regulatory loop in B cells where, after BCR ligation, Lyn phosphorylates tyrosines in immunoreceptor tyrosine-based inhibition motifs (ITIMs) of CD22, permitting the recruitment of SHP-1, which mediates the suppressive effects of CD22 on Ca^{2+} flux and MAPK activation (Chan et al., 1998a; Cornall et al., 1998; Nishizumi et al., 1998; Smith et al., 1998). Cornall et al. (1998) manipulated gene dosage to simultaneously halve the availability of all three components and showed this was sufficient for the development of BCR hyperresponsiveness in *lyn*$^{+/-}$ *CD22*$^{+/-}$*SHP-1*$^{+/-}$ mice. Comparison of B cell development in Lyn- and CD22-deficient mice, however, indicates that only a subset of Lyn's functions are mediated through CD22. For example, both *lyn*$^{-/-}$ and *CD22*$^{-/-}$ mice share loss of MZ B cells (Figure 2) and recirculating B cells in BM but are distinct in peripheral B cell population size (Figure 2). The increase in serum IgM in *CD22*$^{-/-}$ mice is much less than in *lyn*$^{-/-}$ mice. The recent finding that *lyn*$^{up/up}$ primary B cells have constitutive phosphorylation of CD22 and SHP-1 (Hibbs et al., 2002) strengthens the interrelationship between Lyn, CD22, and SHP-1 but suggests constitutive activation of this negative regulatory loop is insufficient to prevent autoimmunity.

CD5. Expression of CD5 defines the B1a subset of B1 cells (Hardy and Hayakawa, 2001). BCR ligation on B1a cells typically induces apoptosis rather than proliferation as occurs in conventional B cells, a response dependent on the presence of CD5 (Bikah et al., 1996). CD5 negatively regulates BCR stimulation via its constitutive association with SHP-1, an association that is Lyn dependent (Ochi and Watanabe, 2000; Sen et al., 1999) and consistent with *lyn*$^{-/-}$ B1a cells proliferating in response to BCR ligation (Ochi and Watanabe, 2000).

Paired Immunoglobulin-like Receptor-B. Expression of paired immunoglobulin-like receptor-B (PIR-B) is restricted to B cells, myeloid cells, and dendritic cells (DCs) (Hayami et al., 1997; Kubagawa et al., 1997). A feature of the structure of PIR-B is the presence of four ITIM-like sequences in the cytoplasmic tail (Maeda et

al., 1998), which appear to mediate the inhibitory activities of PIR-B, including the inhibition of phosphorylation of Syk, Btk, and PLC-γ2 (Maeda et al., 1999), by recruiting SHP-1 and/or SHP-2 (Blery et al., 1998; Yamashita et al., 1998). In mouse macrophages and B cells, tyrosine phosphorylation of PIR-B with the subsequent binding of SHP-1 is constitutive and Lyn dependent (Harder et al., 2001; Ho et al., 1999). B cells from PIR-B$^{-/-}$ mice hyperproliferate mildly in response to BCR ligation and show elevated levels of tyrosine phosphorylation even at rest (Ujike et al., 2002). Surprisingly, conventional B cell development is normal, as are resting serum Ig titers (Ujike et al., 2002). The only B cell developmental abnormality in PIR-B$^{-/-}$ mice is a 2-fold increase in the number of peritoneal B1 cells (Ujike et al., 2002). After immunization with a T cell-dependent antigen, PIR-B$^{-/-}$ mice show an enhanced IgG1 and IgE response (Ujike et al., 2002), although it is not clear if this is B cell intrinsic or requires PIR-B$^{-/-}$ DCs. Interestingly, *lyn*$^{-/-}$ B cells are hyperresponsive to IL4, switching to IgG1 and IgE at 10-fold lower concentrations of IL4 than those required for control B cells (Janas et al., 1999), suggesting possible cooperation between Lyn and PIR-B.

Phosphorylating Adaptor Molecules

Downstream of Tyrosine Kinase. Lyn is required for phosphorylation of p62dok (Dok-1) in B cells (Yamanashi et al., 2000). The downstream of tyrosine kinase (Dok) family of adaptor molecules modulates immunoreceptor, cytokine, and growth factor signaling in a variety of hematopoietic lineages (Lowell, 2004). Inhibition by p62dok is mediated by its phosphorylation-dependent recruitment of RasGAP (Yamanashi and Baltimore, 1997) and Csk (Neet and Hunter, 1995). It is not clear, however, whether the failure to observe phospho-p62dok in *lyn*$^{-/-}$ B cells is due to the direct action of Lyn on p62dok, the failure to phosphorylate either FcγRIIb1 or SHIP-1, or both (see above). MAPK phosphorylation and B cell proliferation are not inhibited in *p62dok*$^{-/-}$ B cells by coengagement of the BCR and FcγRIIb1 as they are in controls but calcium release is (Yamanashi et al., 2000). Little has been reported on the biology of *p62dok*$^{-/-}$ B cells, but serum IgM titers are lower than controls (Yamanashi et al., 2000), suggesting this is Lyn independent. Lyn may also be involved in the activation of Dok-3, which is expressed in B cells and macrophages, phosphorylated by SFK after BCR ligation, and functions to diminish JNK activity through recruitment of SHIP-1 (Lemay et al., 2000; Robson et al., 2004).

Phosphoprotein Associated with Glycosphingolipid-Enriched Microdomains. Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) or Csk binding protein (Cbp) has emerged as a potentially critical regulator of SFK activity through its association with Csk (Brdicka et al., 2000; Kawabuchi et al., 2000). Like Dok, PAG contains several tyrosines that may be phosphorylated and thus serve as potential protein binding sites (Brdicka et al., 2000). Based on analysis of T cells where PAG has been well studied (Brdicka et al., 2000; Torgersen et al., 2001), it is suggested that in the resting state, lipid raft-associated and tyrosine-phosphorylated PAG recruits Csk to the plasma membrane where it phosphorylates the C-terminal tyrosine of SFKs, promoting their inactivation (Hata et al., 1994). Because PAG resides exclusively in lipid rafts where Lyn is the predominant B cell SFK, Lyn may well phosphorylate PAG in B cells. Consistent with this, we find that Lyn coimmunoprecipitates with PAG, and phosphorylation of PAG in *lyn*$^{-/-}$ primary B cells is compromised (our unpublished data). Activation of Lyn after BCR ligation could therefore inhibit the activity of all SFK, including Lyn itself, by recruiting Csk to the rafts. A similar scheme operates in mast cells where Lyn deficiency impairs PAG phosphorylation and Csk recruitment (Odom et al., 2004). It will be of great interest to determine the status of the remaining SFK in *lyn*$^{-/-}$ B cells to ascertain if Lyn deficiency promotes their activation due to defective regulation by PAG and Csk.

Phosphorylating a Negative Regulatory Site on Syk

Syk is an essential positive effector of BCR-stimulated responses (Gauld and Cambier, 2004). After recruitment of Syk to the BCR, it becomes phosphorylated on multiple tyrosines including three (Y-317, -342, and -346) in the linker region separating the tandem SH2 domains from the C-terminal catalytic domain (Keshvara et al., 1998). The phosphorylation of Y-317 creates a binding site for c-Cbl (Yankee et al., 1999), which can serve as a negative regulator of BCR signaling. DT40 B cells lacking Lyn hypophosphorylate Syk Y-317, leading to elevated production of IP3 and an amplified calcium signal (Hong et al., 2002). Although this result needs to be confirmed in other cell systems in which Lyn is not the sole SFK, it broadens our view of how Lyn may negatively regulate BCR signaling to include modulating the turnover of positive signalosomes.

Complementation and Synergy

There have been a limited number of reports of the phenotype of *lyn*$^{-/-}$ mice being modified by genetic crosses and these are summarized below.

lyn x Btk. Given the profound block in B cell activation apparent in B cells lacking Btk, it is not surprising that loss of this kinase dominates the B cell phenotype of *lyn*$^{-/-}$*Btk*$^{-/-}$ mice (Satterthwaite et al., 1998; Takeshita et al., 1998). *lyn*$^{-/-}$*Btk*$^{-/-}$ B cells behave like *Btk*$^{-/-}$ B cells, with low titers of serum IgM and IgG, no proliferation in response to BCR ligation, and loss of B1 cells. Surprisingly, the number of B cells in the spleens of *lyn*$^{-/-}$*Btk*$^{-/-}$ mice is significantly reduced compared to either parent (Satterthwaite et al., 1998; Takeshita et al., 1998), indicating a degree of independence in the signaling pathways controlled by these kinases. Needless to say, *lyn*$^{-/-}$*Btk*$^{-/-}$ mice do not develop autoimmunity, nor do they have the splenomegaly or expanded myeloid population characteristic of Lyn deficiency (Satterthwaite et al., 1998; Takeshita et al., 1998). Another revealing cross introduced a hypomorphic allele of *btk* onto the *lyn*$^{-/-}$*Btk*$^{-/-}$ background (Whyburn et al., 2003). B cells from these mice retained aspects of *lyn*$^{-/-}$ BCR hyperresponsiveness but lost the splenomegaly and expanded myeloid compartment characteristic of *lyn*$^{-/-}$ mice, did not develop significant anti-DNA antibody titers, and had very little serum IgM (Whyburn et al., 2003). This last point suggests that the high frequency of IgM ASC in *lyn*$^{-/-}$ mice is dependent on a process that requires full Btk activity; however, it is unclear what that is and whether it is B cell intrinsic.

lyn x CD19. This cross also prevented the development of the autoimmune disease that afflicts the *lyn*$^{-/-}$ parent (Hasegawa et al., 2001). From the perspective of

B cell biology, the CD19 phenotype was dominant; the *lyn*$^{-/-}$*CD19*$^{-/-}$ mice had increases in IgM levels on peripheral B cells, an absence of peritoneal B1 cells, and T-dependent responses similar to their *CD19*$^{-/-}$ parents (Hasegawa et al., 2001). Lyn deficiency was, however, apparent in the reduced B cell numbers in the periphery, the persistence of splenomegaly, and the expanded splenic myeloid population (Hasegawa et al., 2001). For all intents and purposes, the loss of negative regulation apparent in *lyn*$^{-/-}$ B cells is rendered moot by the loss of signaling due to CD19 deletion.

lyn x fyn. Two groups have made *lyn*$^{-/-}$*fyn*$^{-/-}$ mice. One noted significantly increased mortality due to inheritance of altered kidney physiology from the *fyn*$^{-/-}$ parent combining with autoantibodies from the *lyn*$^{-/-}$ parent to produce a more lethal combination (Yu et al., 2001). Interestingly, this study noted a 10-fold reduction in relative anti-nuclear antibody (ANA) titers in *lyn*$^{-/-}$*fyn*$^{-/-}$ mice compared to the *lyn*$^{-/-}$ parents, which could reflect either the loss of Fyn's positive role in B cell differentiation or an aspect of a Fyn-related T cell defect. The second study found *lyn*$^{-/-}$*fyn*$^{-/-}$ B cells to behave and look like *lyn*$^{-/-}$ B cells with some exceptions (Horikawa et al., 1999). Loss of Fyn reduced *lyn*$^{-/-}$ B cell hyperresponsiveness to BCR ligation, although not to control levels but enough to suggest a positive role for Fyn in BCR signaling in the absence of Lyn (Horikawa et al., 1999). This is interesting in the context of BM-derived mast cells in which Fyn is hyperactive in the absence of Lyn (Odom et al., 2004).

The Role of Lyn-Regulated Pathways in Human Disease

Although modulation of Lyn activity in mice by either genetic ablation or sustained activation results in antibody-mediated autoimmune disease reminiscent of systemic lupus erythematosus (SLE) (Hibbs et al., 2002; Hibbs et al., 1995b; Nishizumi et al., 1995), the role of Lyn in human disease is less clear. SLE is often viewed as an autoimmune disease of B cell hyperactivity (Lipsky, 2001) with many genes implicated in disease susceptibility (Nath et al., 2004; Tsao, 2003). Although altered Lyn expression has been observed in small groups of SLE patients (Huck et al., 2001; Liossis et al., 2001), these results remain difficult to interpret. FcγRIIb1 and CD19, identified in mice both as targets of Lyn and as predisposing to autoimmune disease when mutated (Bolland et al., 2002; Tedder et al., 1997), have been implicated in human disease development. Augmented CD19 signaling is implicated in systemic sclerosis (Sato et al., 2000), and polymorphisms in FcγRIIb1 are identified as heritable risk factors for SLE (Kyogoku et al., 2002; Su et al., 2004). In addition, mutations in the *HS1* gene, the product of which is a substrate of Lyn (Yamanashi et al., 1993), have also been observed in SLE patients (Otsuka et al., 2004; Sawabe et al., 2003). Although these results are suggestive, more extensive work on large subject groups is required for Lyn itself to be considered an SLE candidate gene. Rather than individual human patients possessing single genetic changes that confer constitutive Lyn activity or inactivity, it appears more likely that such people will have perturbations in steps along Lyn-regulated pathways that collectively may have an effect similar to that observed in the genetically modified mice.

Concluding Remarks

Lyn is the predominantly expressed SFK in B cells and has both positive and negative regulatory roles in BCR-induced signal transduction. Accumulating evidence indicates that the positive functions of Lyn after BCR ligation are probably context dependent, such that Lyn can be substituted by other SFK depending on the nature of the stimulation. The irreplaceable function of Lyn in B cells is to set the threshold of negative feedback control of signaling after BCR ligation and this is achieved by multiple mechanisms that may work synergistically and independently. Important future challenges are to verify in vitro findings with in vivo models in order to explain how loss of Lyn leads to the production of high titer autoantibodies when transgenic model systems suggest that Lyn deficiency enhances the induction of B cell tolerance. The role of Lyn in establishing signaling thresholds in other lineages of hematopoietic cells and the role of these cells in the development of autoimmune disease may provide valuable insights into autoimmune disease etiology. These studies may well contribute not only to the knowledge of B cell biology but also to the development of pharmaceutical reagents for better management of human diseases that may have dysregulated Lyn-related pathways as contributing or causative factors.

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